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Kainate Receptor Subunits Expressed in Single Cultured Hippocampal Neurons: Molecular and Functional Variants by RNA Editing

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Summary

To determine the kainate receptor subunits that are found in native kainate receptors, we have applied a multiplex PCR of cDNAs reverse transcribed from mRNA harvested from single cultured hippocampal neurons after electrophysiological recording. We found that all the cells showing rapidly desensitizing currents in response to kainate express the GluR6 subunit mRNA, and that some of them also express the GluR5 subunit mRNA. No GluR7, KA-1, or KA-2 subunit mRNAs were detected. Analysis of the editing sites of the GluR6 mRNA demonstrated that the three editing sites present in these subunits are edited to a different extent. Predominant expression of the unedited variant (Q) was observed, but edited and unedited variants may coexist in the same cell. In addition, we show that the Q/R site from the GluR6 subunit controls functional properties of native kainate receptors.

Introduction

The non-NMDA ionotropic glutamate receptors have been classified into two different groups according to their binding affinity for the agonists α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainate (Monaghan et al., 1989; Watkins et al., 1990). Five subunits, named GluR5 (Bettler et al., 1990), GluR6 (Egebjerg et al., 1991), GluR7 (Bettler et al., 1992), KA-1 (Werner et al., 1991), and KA-2 (Herb et al., 1992), have been demonstrated to generate glutamate receptors with high affinity for kainate in expression systems (Bettler et al., 1992; Herb et al., 1992; Lomeli et al., 1992; Werner et al., 1991). However, only the GluR5 and GluR6 subunits can form functional channels when expressed in vitro (Egebjerg et al., 1991; Sommer et al., 1992; Köhler et al., 1993). The mapping of the mRNAs for these subunits in the central nervous system by in situ hybridization has revealed a differential distribution, with GluR6 and KA-2 being the most widely expressed (Wisden and Seeburg, 1993).

One source of potential heterogeneity in glutamate receptor subunits is RNA editing. Editing of RNA is a phenomenon in which a single nucleotide exchange between the DNA and RNA sequences occurs, originating molecular variants of the same subunit. In three glutamate receptor subunits, the AMPA receptor subunit GluR2 (GluR-B; Keinänen et al., 1990; Boulter et al., 1990; Nakanishi et al., 1990) and the kainate receptor subunits GluR5 (Bettler et al., 1990) and GluR6 (Egebjerg et al., 1991), a single nucleotide exchange as a result of RNA editing determines the presence of a glutamine (Q) or arginine (R) residue in the Q/R site located in the second transmembrane domain (Sommer et al., 1991). Recently, two additional sites generated by editing, named I/V and Y/C, located in the first transmembrane domain have also been described in the GluR6 subunit (Köhler et al., 1993). These three residues are critical determinants of calcium permeability and rectification properties of kainate receptors as seen in homomeric GluR6 channels expressed in host cells (Köhler et al., 1993; Egebjerg and Heinemann, 1993). In particular, the editing of the Q/R site in the GluR6 subunit seems to govern the rectification properties in homomeric GluR6 channels (Herb et al., 1992; Köhler et al., 1993). The unedited version encodes a glutamine and exhibits strong inward rectification, whereas the edited version encodes an arginine at this position and does not rectify (Egebjerg et al., 1993). However, calcium permeability seems to be controlled by the Q/R site whenever both the I/V and Y/C sites are edited (Köhler et al., 1993).

Native kainate receptor channels have been characterized only recently, and little is known about their subunit composition (see Feldmeyer and Cull-Candy, 1994). Rapidly desensitizing responses upon kainate application were first described in the peripheral nervous system (Huettner, 1990) and more recently in hippocampal neurons (Lerma et al., 1993) and glia (Patneau et al., 1994). The subunit composition of these kainate receptors in cultured hippocampal neurons is unknown. Recently, a combination of whole-cell patch clamp recording and analysis by polymerase chain reaction (PCR) in single identified neurons has been used to address the question of the molecular composition of native glutamate receptors (Lambolez et al., 1992; Audinat et al. 1994; Bochet et al., 1994; Jonas et al., 1994). This method was modified to characterize, from a molecular point of view, native glutamate receptors of the kainate type. Following a multiplex PCR (mPCR) protocol, all the kainate receptor subunits were independently tested from the same cell. We have also analyzed RNA editing in the PCR-generated GluR6 fragments derived from these cells in correlation with their functional properties.

Results

mPCR

The mPCR assay was designed to amplify the five different kainate receptor subunits (GluR5–7 and KA-1 and KA-2) expressed in single cells. For this purpose, a first round of PCR mixing the four specific primer pairs for GluR5, GluR6, GluR7, KA-1, and KA-2 (these subunits were ampli-

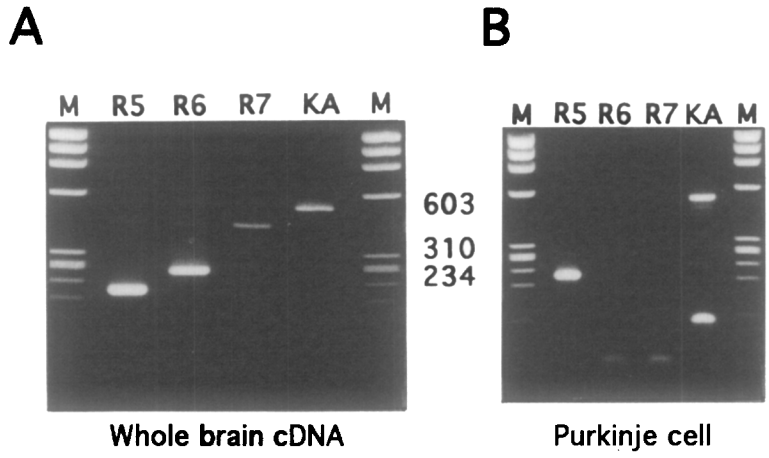

Figure 1. Kainate Receptor Subunits Present in Whole Rat Brain and Purkinje Cells

(A) cDNA reverse transcribed from RNA extracted from whole brains of 21-day-old rats was used (0.5 ng) as a template to amplify the high affinity kainate receptor subunits, using the mPCR protocol (see Experimental Procedures). The 208, 259, 421, and 512 bp bands correspond to the GluR5, GluR6, GluR7, and KA-1/KA-2 PCR-generated fragments, respectively.

(B). The same protocol was applied to RNA harvested from a cerebellar Purkinje cell. The molecular weight markers are as in (A). Note the absence of amplification of GluR6 and GluR7 subunits in cerebellar Purkinje cell. Two other Purkinje cells were analyzed with similar results.

Lanes M in (A) and (B), FX174 HaeIII molecular weight markers.

fied using the same pair of primers) subunits was performed. The PCR products were subsequently purified and used in a second round of PCR as a template to amplify the different subunits. In this second round, each subunit was amplified independently using its specific pair of primers, resulting in four PCR products corresponding to GluR5, GluR6, GluR7, KA-1, and KA-2 cDNAs, respectively. To evaluate the reliability of this assay, mPCR was used to amplify the five different kainate receptor subunits using RNA purified from adult rat brain. Figure 1A is an ethidium bromide–stained gel demonstrating that all of the kainate receptor subunits were amplified when cDNA from whole rat brain was used as a template. Fragments of the predicted size were obtained, and they were identified by Southern blot analysis with specific probes and specific restriction enzymes: BbvI, MaeII, AluI, EcoRI, and KpnI for GluR5, GluR6, GluR7, KA-1, and KA-2 subunits, respectively (data not shown). The mPCR assay was then used to amplify specific kainate receptor subunits expressed in single Purkinje cells from rat cerebellar slice cultures. As expected from in situ hybridization results in Purkinje cells (Wisden and Seeburg, 1993), neither GluR6 nor GluR7 were detected, but GluR5, KA-1, and KA-2 subunit mRNAs were observed (Figure 1B). Thus, all of the kainate receptor subunits in whole rat brain, as well as specific subunits expressed at the single cell level, could be amplified by the mPCR protocol.

Kainate Receptor Subunits Expressed by Single Hippocampal Cells

The electrophysiological properties of the glutamate receptors of the kainate subtype in single cultured neurons

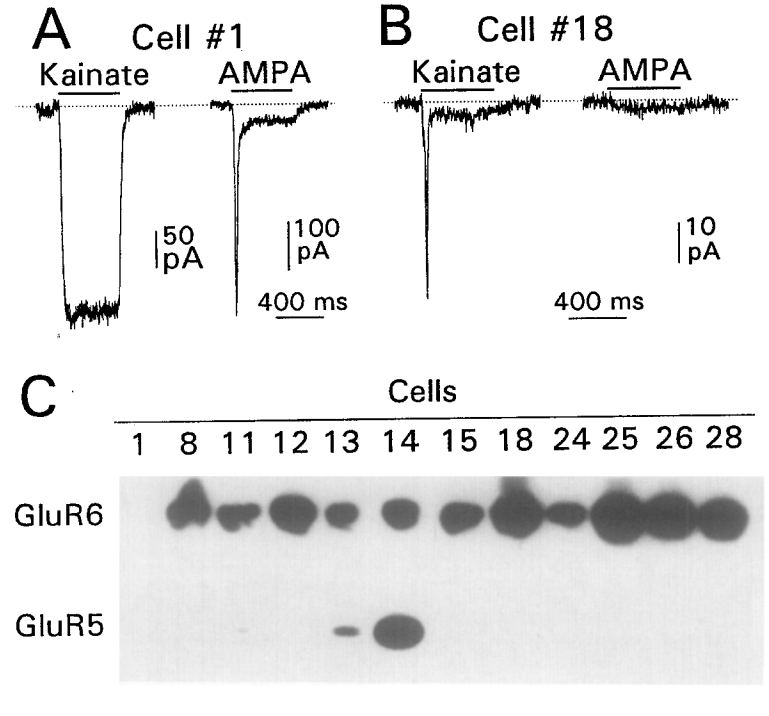

Figure 2. Responses to Kainate and Southern Blot Analysis of Glutamate Receptor Subunits in Cultured Hippocampal Neurons

(A and B) Whole-cell currents evoked by rapid application of kainate (300 μM) and S-AMPA (200 μM) are illustrated for two cultured hippocampal cells voltage-clamped at −60 mV, which were subjected to RT-mPCR analysis. The duration of agonist application is indicated by the bar above each trace. Note the lack of response to AMPA of the cell shown in (B).

(C) The cDNA fragments obtained after RT-mPCR corresponding to the GluR5–7 and KA-1 and KA-2 subunits from 11 cultured hippocampal cells, responding to kainate as in (B), were resolved on four agarose gels, transfered onto four nylon membranes, and hybridized with the GluR6-, GluR5-, GluR7-, and KA-1/KA-2-specific probes. All cells expressed the GluR6 subunit (first row), and in some of them GluR5 was also detected (second row). No signal was obtained with GluR7 and KA-1/KA-2 probes in any cell (data not shown). Cell 1 did not respond to kainate with transient currents, but with responses of the AMPA receptor-mediated type (A). No signal corresponding to the GluR5–6 (column 1) nor GluR7, KA-1, and KA-2 subunit mRNAs (data not shown) were detected in this and in two additional cells with similar electrophysiological characteristics.

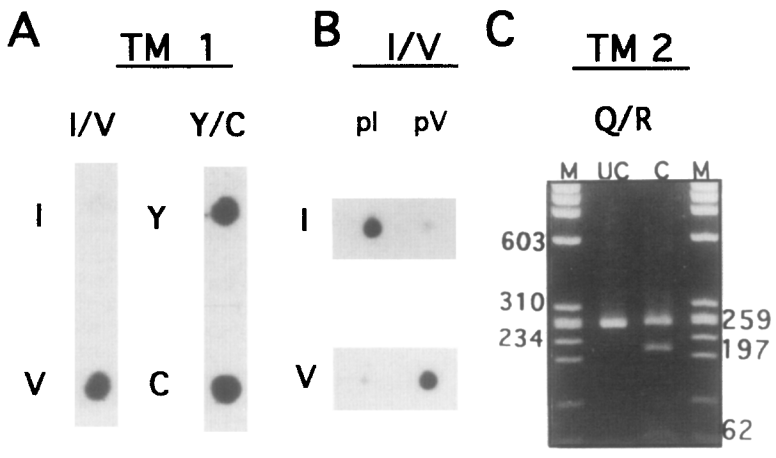

Figure 3. RNA Editing Analysis of PCR-Amplified GluR6 Subunits in a Single Cultured Hippocampal Neuron

(A) cDNA fragments (1 ng) were fixed onto a nylon membrane and hybridized with the specific ^{32}P-labeled oligoprobes for the edited (V and C) or unedited variants (I and Y) for the I/V and the Y/C sites, respectively.

(B) For the I/V site, recombinant plasmid carrying the GluR6 fragment either edited (pV) or unedited (pI) were included as controls.

(C) Agarose gel electrophoresis of the amplified cDNA fragment corresponding to the GluR6 subunit (lane UC), and the same fragment after incubation with the AciI restriction enzyme (lane C). The AciI restriction enzyme cuts the edited version (R) and does not cut the unedited version (Q). The presence of Q variant is indicated by the 259 bp band, resistant to AciI, and the R variant by the generation of two bands of 197 and 62 bp (lane C). Lanes M, FX 174 HaeIII molecular weight markers. The examples shown in this figure correspond to the analysis of cell 15.

were characterized using whole-cell patch-clamp recording in combination with a rapid perfusion system (Lerma et al., 1993). In all these cells, a fast desensitizing inward current was detected upon rapid kainate application (300 μM), and no response or a negligible response was observed upon rapid AMPA application (200 μM; Figure 2B), indicating that these neurons express mainly kainate receptors. To determine which of the kainate receptor subunit mRNAs were present, the cytoplasm of each cell was aspirated into the recording pipette, and mPCR for GluR5, GluR6, GluR7, KA-1, and KA-2 glutamate receptor subunits was performed on cDNAs prepared from these cells. The PCR products were separated by agarose gel electrophoresis, transferred onto a nylon membrane, and analyzed by Southern blot using specific radiolabeled probes. The results obtained from 11 cells responding to application of kainate with rapidly desensitizing responses are shown in Figure 2C. In all the cells, the fragment corresponding to the GluR6 subunit–specific mRNA was observed, and in 3 of 11 cells analyzed, the GluR5 mRNA was also detected (cells 11, 13, and 14). In contrast, no signal corresponding to the GluR7, KA-1, and KA-2 subunit–specific mRNAs were detected in any of the 11 cells examined (data not shown). To see whether there was a correlation between the electrophysiological response and the pattern of mRNA expression, the same mPCR protocol was also applied to 3 cells that did not show desensitizing responses upon kainate application but ex-

Table 1. Edited and Unedited Variants of the GluR6 Subunit Expressed in Single Cultured Hippocampal Neurons.

	TM1		TM2	
Cell	I/V	Y/C	Q/R	IR
8	−/+	+/+	+/+	ND
14	−/+	+/+	+/+	ND
15	−/+	+/+	++/+	0.07
18	−/+	+/+	+/++	ND
24	++/+	+/+	+/−	0.06
25	−/+	+/+	++/+	0.14
26	−/+	+/+	++/+	ND
28	ND	ND	−/+	0.8
29	ND	ND	+/−	0.0
31	ND	ND	+/−	0.08
36	ND	ND	++/+	0.31
40	ND	ND	+/+	0.37
42	ND	ND	+/−	0.0
44	ND	ND	+/−	0.0
46	ND	ND	+/−	0.0
47	ND	ND	+/−	0.0
48	ND	ND	+/−	0.0
50	ND	ND	+/−	0.2
51	ND	ND	+/−	0.0

The presence of edited and unedited variants for the I/V and Y/C sites (first transmembrane domain [TM1]) were assayed by Southern blot analysis. The Q/R site (second transmembrane domain [TM2]) was assayed by digestion with the AciI restriction enzyme. IR is the index of rectification of kainate-induced currents calculated as the ratio of slope conductance at +40 and −60 mV. (+/−), detected/nondetected; (+/+), both variants detected at similar levels; (+/++, ++/+), both variants detected, one of them at higher level. ND, not determined.

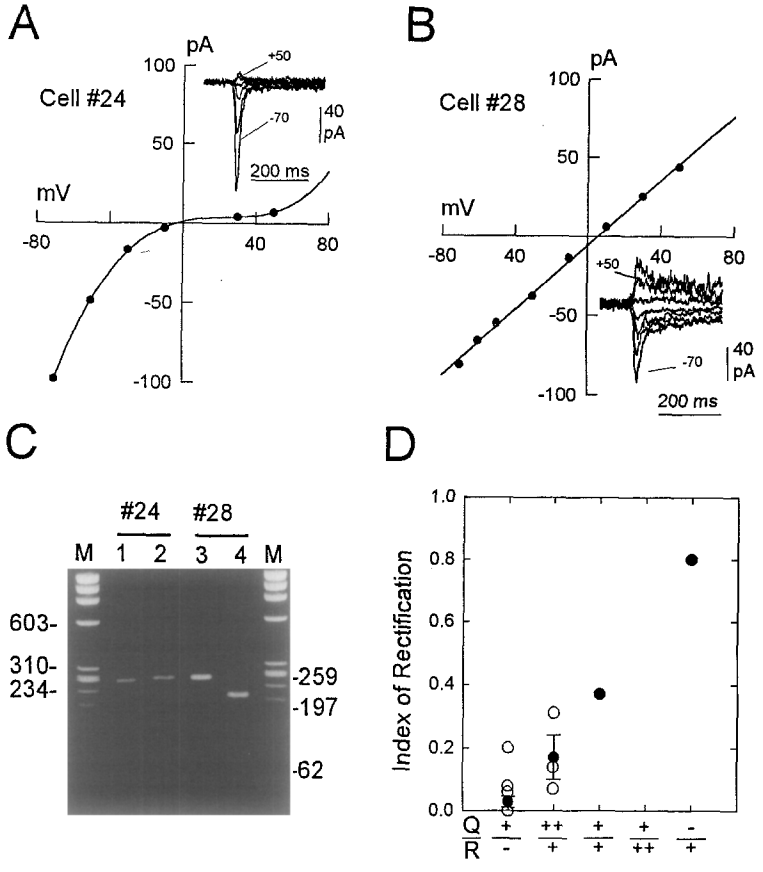

Figure 4. Correlation of Functional Properties of Native Kainate Receptor Channels and RNA Editing in the Q/R site of the GluR6 Subunit in Cultured Hippocampal Neurons

(A and B) Current–voltage relationships of the kainate-induced responses in two different cultured hippocampal neurons, cell 24 showing strong inward rectification (A) and cell 28 with an approximately linear current–voltage relationship (B). Current–voltage relationships were made by rapidly applying kainate (300 μM) while holding the membrane potential at different voltages (from −70 mV to +50 mV).

(C) RNA editing analysis of Q/R site of the GluR6 fragment PCR-generated from cells 24 and 28. The GluR6 fragments were resolved by agarose gel electrophoresis. Lanes M, FX 174 HaeIII molecular weight markers; lanes 1 and 3, GluR6 fragments from cells 24 and 28, respectively; lanes 2 and 4, same fragments after incubation with the AciI restriction enzyme. Note the absence of digestion in cell 24 and the full digestion in cell 28 (lanes 2 and 4), indicating the exclusive presence of Q and R variants, respectively.

(D) The relative amount of edited mRNA was estimated as a function of unedited mRNA and plotted against the index of rectification . Symbols are as in Table 1. Open points are single values. Closed points are the mean ± SEM of 10 cells (+/-), 3 cells (++/+), 1 cell (+/+), and 1 cell (-/+).

pressed AMPA receptors. In these cells, kainate induced a large steady current, whereas AMPA induced a typical desensitizing response (Figure 2A). Figure 2C, lane 1, shows the result obtained from one of these cells. No signal corresponding to the GluR5, GluR6, or GluR7 and KA-1 and KA-2 subunit mRNAs was detected. These results demonstrate that the neurons showing rapidly desensitizing responses to kainate application contained GluR6 subunit mRNA, and that some of them express a heterogeneous population of GluR5 and GluR6 subunits.

Editing of the GluR6 Subunit mRNA

Since the expression of the GluR6 subunit was detected in all the cells analyzed, we investigated the three sites targeted by RNA editing present in this subunit. These sites have been shown to control the calcium permeability and the rectification properties of homomeric GluR6 receptors channels (Herb et al., 1992; Köhler et al., 1993). In a first round, we analyzed the three sites of editing in a set of 8 cells previously characterized (see above). To correlate the rectification properties with the RNA editing of the Q/R site (see below), we extended the analysis of this site to a total of 19 cells. For the I/V and Y/C sites, the different molecular variants generated by editing were analyzed by probing with specific oligonucleotides designed to detect the single nucleotide exchange. For the Q/R site, this was assessed by using the endonuclease AciI (see Experimental Procedures for more details). Figure 3 shows a representative example of GluR6 RNA editing analysis in cell 15. In this cell, the edited variant (V) for the I/V site was exclusively detected, and similar levels of expression of the edited (C) and unedited (Y) variants for the Y/C site were observed (Figure 3A). Predominant expression of the unedited variant (Q) for the Q/R site was also observed (Figure 3C). A similar analysis was made in a total of 8 cells, and different RNA editing patterns were observed for the three sites, as shown in Table 1. In the I/V site, the edited variant (V) was exclusively observed in the majority of the cells (7 out of 8 cells) and the unedited variant (I) was detected in only 1 cell (cell 24). In contrast, similar levels of both variants were detected for the Y/C site in all cells analyzed. The Q/R site was analyzed in 19 cells, and the unedited variant (Q) predominated in 14 cells (74%), being the only variant detected in 10 cells. In contrast, the edited variant (R) was more abundant in 2 cells (10%), one of which expressed only this variant (cell 28; see Table 1).

Editing in the Q/R Site and Current-Voltage Relationship

The rectification properties of homomeric GluR6 channels seem to be controlled by the Q/R site. We have examined this relationship in single cultured neurons from the hippocampus expressing native kainate receptors. For this purpose, current–voltage relationships of kainate-induced peaks were made, and the PCR-generated GluR6 frag-

ment was analyzed for RNA editing in the Q/R site coding region. Peak currents activated by kainate presented marked inward rectification in 14 out of 15 cells (93%) subjected to RT-PCR analysis. Four cells (27%) (cells 25, 36, 40 and 50) showed some transient outward current at inside positive potentials, and there was only 1 cell (6%) showing a linear peak current–voltage relationship (cell 28). These results are in accordance with previous reports showing mostly inward rectifying current–voltage relationships for kainate-activated peak currents in cultured hippocampal neurons (Lerma et al., 1993). The RNA editing analysis of the Q/R site showed that in 10 cells the mRNA encoded exclusively for a glutamine, whereas in 1 cell (cell 28) the mRNA was edited predicting an arginine residue. Figure 4 shows the current–voltage relationships obtained from cells 24 and 28, demonstrating a strong inward rectification of the peak current (cell 24; Figure 4A) and an almost linear peak current–voltage curve (cell 28; Figure 4B), respectively. The editing analysis of the Q/R site in both cells, performed by cutting the PCR-generated GluR6 fragment with the AciI restriction enzyme (see Experimental Procedures), showed the absence of cut in the fragment corresponding to the GluR6 subunit cDNA from cell 24, indicating the lack of editing in the Q/R site coding region in this cell (Figure 4C). In contrast, the PCR-generated fragment from cell 28 was cut by this enzyme, indicating the complete editing of the Q/R site of GluR6 cDNA from this cell (Figure 4C). Of the cells in which the I/V relationship was calculated, we also found 3 cells (cells 15, 25, and 36) in which the mRNA for the edited variant was at lower level than the unedited form (Figure 3C shows an example for cell 15) and 1 cell (cell 40) in which both forms were observed at similar levels (Table 1). Rectification properties were directly correlated with the relative presence of edited and unedited variants in a single neuron. To quantitate rectification, the slope conductance was measured at +40 and −60 mV. The ratio of conductances (G_{+40}/G_{-60}) was taken as the index of rectification (see Lerma et al., 1994). The correlation was demonstrated by plotting the index of rectification versus the ratio of unedited (Q) to edited (R) mRNAs in these cells (Figure 4D).

In conclusion, these results demonstrate that the molecular heterogeneity generated by GluR6 RNA editing influences functional properties of native kainate receptors, as previously reported for homomeric GluR6 channels expressed in heterologous systems (Köhler et al., 1993; Egebjerg and Heinemann, 1993).

Kainate Receptors Are Affected by the Dye Evans Blue

By performing electrophysiological measurements of glutamate receptors expressed recombinantly in Xenopus oocytes, Keller et al. (1993) have identified a noncompetitive antagonist that shows subunit specificity. The Evans blue dye (a biphenyl derivative of naphthalene disulfonic acid) blocks combinations of AMPA receptor subunits but does not block GluR6 homomeric formations (up to 3 μM). If kainate receptors in hippocampal cells are formed exclusively by GluR6, desensitizing responses to kainate are expected to be insensitive to Evans blue antagonism. To test this hypothesis, kainate was rapidly applied to cells with a pure population of kainate receptors in the absence or presence of Evans blue. Surprisingly, the peak current evoked by kainate was reduced by 75% (25% ± 5.9% of the control response remained; n = 7) in the presence of 3 μM Evans blue (Figure 5A), whereas at 100 μM this current was blocked completely (data not shown; n = 3). In both cases, recovery was only partial. In our studies, we have observed that in about 20% of the cells displaying desensitizing responses to kainate, the onset of desensitization is best fitted by the sum of two exponentials (Lerma et al., 1993). In these cells, the effect of Evans blue was totally contrary to the responses showing single exponential decays; the desensitization was slowed and a substantial steady current did appear at equilibrium. A representative example is illustrated in Figure 5B.

Different sensitivity to Evans blue did not correlate with expression of particular kainate receptor subunits at the single cell level. RT-PCR analysis of the mRNA harvested from neurons in which the kainate-activated response was either inhibited or potentiated showed a similar expression pattern. For example, the two representative cells shown in Figure 5 correspond to cell 15 (potentiated by Evans blue; Figure 5B) and cell 24 (blocked by Evans blue; Figure 5A). The expression pattern of kainate receptor subunits

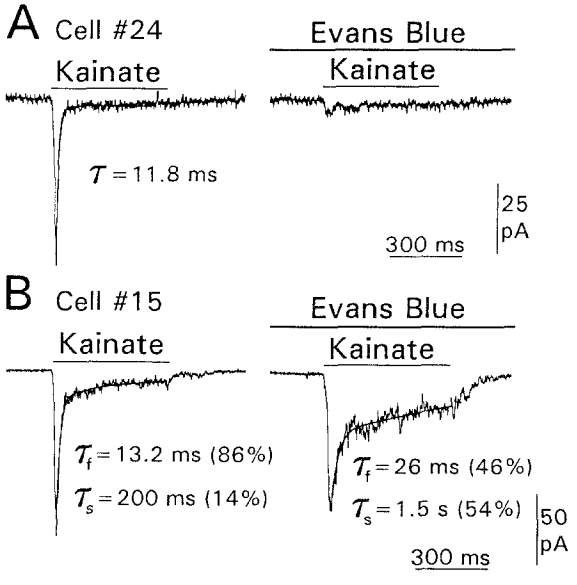

Figure 5. Effects of Evans Blue on Native Kainate Receptors

(A) A cell showing a single exponential decay (superimposed solid line; time constant indicated) of desensitization onset (left record). Evans blue (3 μM) blocked the kainate-induced transient current (right record).

(B) A cell in which onset of desensitization followed a double exponential time course. In this type of cells, Evans blue (3 μM) slowed the rate of desensitization and increased the steady response (right record). The superimposed solid lines represent the fit to the sum of two exponentials (time constant values and relative amplitude indicated). In both cases, kainate was at 300 μM. Cells shown in (A) and (B), corresponding to cells 24 and 15, respectively, were subjected to RT-PCR analysis.

in both cells is illustrated in Figure 2C (columns 7 and 9, respectively). GluR6 was exclusively expressed by both neurons, and no evidence for expression of GluR5, GluR7, KA-1, and KA-2 was found in these neurons or in similar cases.

Discussion

mPCR

In the present study, we have determined the kainate receptor subunit–specific mRNAs present in cultured hippocampal neurons that are selectively activated by kainate. These glutamate receptors have previously been characterized electrophysiologically (Lerma et al., 1993; Paternain et al., 1995), and now mPCR has allowed us to analyze their molecular composition. Our results demonstrate a major expression of the kainate receptor subunit GluR6 mRNA in all the cells examined. Whereas there was no evidence of expression of GluR7, KA-1, or KA-2, GluR5 mRNA was detected in 3 cells. It could be argued that with this mPCR protocol, the GluR6 subunit mRNA was amplified more efficiently than the other subunit mRNAs. However this seemed not to be the case for the following reasons. First, all the kainate subunit mRNAs were detected when cDNA from whole rat brain was used as a template (see Figure 1A). Second, at the single cell level, GluR5, KA-1, and KA-2 subunit mRNAs were efficiently amplified from Purkinje cells. This pattern of expression contrasted with hippocampal neurons, in which the KA-1 and KA-2 subunit mRNAs were not detected and GluR5 subunit mRNA was observed at high levels in only one cell (see Figure 2C). In contrast, no signal was obtained in Purkinje cells for GluR6 subunit mRNA, which was detected in all the hippocampal neurons tested and selected according to their electrophysiological response.

Subunit Composition of Native Kainate Receptors

We have found that the majority of cultured hippocampal neurons that are activated by kainate only express the GluR6 kainate receptor subunit mRNAs. Interestingly, in 3 of 11 cells, GluR5 mRNA was also detected (see Figure 2C). However, with one exception, the responses of these cells to kainate application were similar to the other cells, suggesting that the GluR5 receptors expressed by these hippocampal neurons are a minor component of the rapidly desensitizing kainate-induced response. Comparison of the electrophysiological properties of transfected homomeric GluR6 receptors (Egebjerg et al., 1991; Herb et al., 1992; Köhler et al., 1993) with those expressed by single hippocampal neurons in culture (this study; Lerma et al., 1993), together with the high levels of GluR6 mRNA observed in hippocampus (Wisden and Seeburg, 1993), indicate that kainate receptors expressed by these cells are most likely native homomeric GluR6 glutamate receptors. From functional expression studies, it is known that GluR6 and KA-2 subunits can coassemble to generate glutamate receptors that are activated by kainate and by AMPA (Herb et al., 1992). High levels of KA-2 subunit mRNA have also been detected in hippocampus (Wisden and Seeburg, 1993). However, in the hippocampal cells analyzed here showing completely desensitizing kainate-induced responses, AMPA failed to evoked a current (e.g., see Figure 2B), and, consistent with the electrophysiological results, we failed to detect the KA-2 subunit mRNA in any of the cells examined (see Figure 2C). Finally, these kainate receptors seem to be different both at the electrophysiological and molecular levels from the peripheral kainate receptors described in dorsal root ganglion, which are activated by kainate and AMPA (Huettner, 1990) and in which the GluR5 subunit has been proposed to be the major component (Sommer et al., 1992).

Editing of GluR6 RNA

RNA editing has been proposed as a source of molecular heterogeneity in a variety of mRNAs encoding for several proteins (Cattaneo, 1991; see Sommer and Seeburg, 1992 for review). In the mammalian brain, the GluR6 subunit presents three positions that can be diversified by RNA editing, generating eight possible variants with different functional properties when expressed in homomeric formations (Sommer et al., 1991; Köhler et al., 1993). An important question is whether different molecular variants of the GluR6 subunit are coexpressed by a single cell or whether edited and unedited subunits do not coexist in the same cell. The analysis of RNA editing of the GluR6 subunit in single cultured hippocampal neurons demonstrated that different edited variants are coexpressed by a single cell. In addition, the extent of RNA editing was site dependent, showing clear differences among the three sites. In most cells, the edited variant at the I/V site was expressed exclusively, whereas the Q/R site was mostly unedited (Table 1). Edited GluR5 subunits at the Q/R site have also been found to be uncommon in single hippocampal neurons (Mackler and Eberwine, 1993). In contrast, all cells studied showed edited and unedited mRNAs at the Y/C site to a similar extent. These results demonstrate that different molecular variants generated by RNA editing of the GluR6 subunit are not expressed randomly in single neurons and suggest the existence of different site- and cell-specific regulators of editing, resulting in a controlled distribution of edited and unedited GluR6 glutamate receptor subunits. RNA editing in the GluR6 subunit has also been demonstrated to have functional consequences in homomeric, recombinantly expressed GluR6 channels. The calcium permeability of the GluR6 channels is modulated by the Q/R site, located in the second transmembrane domain, whenever the I/V and Y/C sites, located in the first transmembrane domain, are edited (Köhler et al., 1993). However, the rectification properties seem to be exclusively controlled by the Q/R site in the same way as for the AMPA receptors (Herb et al., 1992; Egebjerg and Heinemann, 1993; Köhler et al., 1993). The unedited version encodes a glutamine and exhibits strong inward rectification, whereas the edited version encodes an arginine at this position and does not rectify (Egebjerg and Heinemann, 1993). Our results demonstrate a clear relationship between the rectification properties of native kainate receptors and RNA editing of Q/R site in the GluR6 subunit RNA from single neurons. A linear current–voltage relationship was observed when

edited forms (R) predominated, whereas inward rectification was associated with the expression of unedited (Q) RNA. These results demonstrate that the molecular heterogeneity resulting from RNA editing does indeed have physiological consequences in naturally occurring glutamate receptors. The data also suggest that the rectification properties are exclusively controlled by editing of the Q/R site and are independent of editing sites of the first transmembrane domain coding region. Although we have mostly found cells expressing the unedited version of the Q/R site and inwardly rectifying current–voltage relationships, we also found some cells expressing both editing variants, which showed some outward current at inside positive potentials. The correlation found between the relative levels of edited and unedited GluR6 mRNA and the degree of rectification of kainate-induced currents indicates not only that native kainate receptors should be made up of GluR6 subunits, but also that the proportion of kainate receptors having or lacking edited subunits in a given cell determines the rectification properties of the whole cell.

Finally, the GluR6 (I, Y, R) and GluR6 (V, C, R) subunits have been shown to have a moderate calcium permeability when assembled into homomeric channels. However, when heteromeric receptors from these and any of the GluR6 variants are assembled, the calcium permeability is markedly reduced (Köhler et al., 1993). Although we have not determined calcium permeability in cells subjected to mRNA analysis, from our results, we might expect a mosaic of heteromeric GluR6 channels in most, if not all, hippocampal cells, which would result in a low calcium permeability. Actually, analyses of calcium permeability in cultured hippocampal neurons similar to those used in this study indicate a very low permeability to calcium for these channels, even when current–voltage relationships show marked inward rectification (Lerma et al., 1993). This means that, in contrast to the AMPA receptors (see Lerma et al., 1994), calcium permeability and rectification properties are independent in native kainate receptors.

Evans Blue Modulation of the Kainate Receptor-Mediated Responses

Although RT-PCR data suggest that native kainate glutamate receptors are made up from GluR6 subunits, functional properties suggest that another still unknown factor(s) might be taking part of the functional assembly, giving rise to GluR6 receptor channels with different functional properties. The feature that differentiates the channels formed recombinantly in oocytes by GluR6 subunits and hippocampal kainate receptors is that the dye Evans blue blocked kainate receptor–mediated responses in a population of cells, while decreasing the desensitization and potentiating the current in other cells. Recent experiments on oocytes expressing GluR6 indicate that these receptors are not sensitive to Evans blue (Keller et al., 1993). Although it was not studied systematically, the sensitivity to Evans blue could not be linked to GluR6 editing sites either. Such a distinction between homomeric GluR6 and native kainate receptors indicates that more than a single configuration of kainate receptors might be expressed by hippocampal cells. The common characteristic among the responses inhibited by Evans blue was that the onset of desensitization was monoexponential. In contrast, only those responses showing biexponential desensitization kinetics were potentiated by this compound. The disparate effect of Evans blue on different cells suggests functional diversity among kainate receptors in the hippocampus. In fact, there are other cloned glutamate receptor subunits that are orphan, since modification of the functional properties of receptor channels formed by other subunits has not been demonstrated. For instance, δ1 and δ2 subunits present 22%–28% homology with non-NMDA receptor subunits (Yamazaki et al., 1992; Lomeli et al., 1993). Alternatively, still unknown subunits for glutamate receptors may exist. This is reminiscent of other ligand- or voltage-gated channels. For instance, currents produced by expression of potassium channel subunit clones do not show identical properties to those recorded in native cell membranes. The isolation of cDNAs coding for β subunits of voltage-gated calcium and potassium channels provides a possible explanation for this discrepancy. Calcium channel β subunit determines the magnitude of the current and changes the kinetics of calcium channels (see Pragnelli et al., 1994). Similarly, recent cloning of the β subunit for potassium channels demonstrated its modulatory action on functional properties of these channels (Scott et al., 1994; Aldrich, 1994).

In summary, rapidly desensitizing responses to kainate are associated with selective expression of GluR6 subunits in single hippocampal cultured neurons. The RNA encoding for this glutamate receptor subunit is edited to a different extent in three sites; one of them, the Q/R site, controls rectification properties of naturally occurring channels.

Experimental Procedures

Cell Culture

Cell cultures were prepared from hippocampi of embryonic day 18 rats as described previously (Lerma et al., 1993). In brief, hippocampal neurons were mechanically dissociated from 17- to 18-day-old rat embryos after treatment with trypsin I (0.12 mg/ml, 15 min, 37°C; Sigma) and seeded onto 35 mm Petri dishes previously coated with poly-D-lysine (5 mg/ml) and laminin (4 μg/ml). The initial density was adjusted to 10^3 cells/mm^2. Cells were incubated in Dulbecco's modified Eagle's medium supplemented with transferrin (0.1 mg/ml), insulin (5 μg/ml), putrescine (100 μM), progesterone (20 nM), SeO_2 (30 nM), ovalbumin (0.1%), glucose (3.3 mM), sodium pyruvate (1 mM), glutamine (4 mM), and antibiotics in a humidified incubator at 37°C and 5% CO_2.

Recordings and Perfusion Procedures

Electrophysiological experiments were carried out 1–4 days after plating. Membrane currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) using a List EPC-7 amplifier. Recording pipettes had a tip resistance of 3–4 MΩ and were filled with 8 μl of the following autoclaved solution: 130 mM CsCl, 4 mM $MgCl_2$, 2 mM EGTA, and 10 mM HEPES (buffered to pH 7.5 with CsOH [310 mOsm]). In some occasions (cells 29–51), Na^+ substituted for Cs^+. The external solution was composed of 160 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (buffered to pH 7.5 with NaOH [330 mOsm]). The cells were rapidly perfused using a linear array of 6–8 glass tubes placed 200–300 μm from the soma. Ringer's solution with and without agonist flowed from adjacent barrels. Solution changes were achieved by later-

ally displacing the whole perfusion array, using a motorized device controlled via a personal computer (see Lerma, 1992).

Kainate was from Sigma, and AMPA, from Tocris Neuramin. Evans blue (kindly provided by Dr. Martinez-Murillo, Instituto Cajal) was from Sigma.

At the end of the recording, the content of the cell was aspirated into the recording pipette and expelled into a tube. Subsequently, reverse transcription was performed in a final volume of 10 μl, as described in Lambolez et al. (1992).

mPCR Amplification

Following reverse transcription, the GluR5, GluR6, GluR7, KA-1, and KA-2 subunits were amplified using the following set of primers (from 5′ to 3′): GluR5 sense, GCCCCTCTCACCATCACATAC (position 1587, position 1 being the first base of the initiation codon of GluR51a [Bettler et al., 1990]); GluR5 antisense, ACCTCGCAATCACAAACAGTACA (position 1795); GluR6 sense, TTCCTGAATCCTCTCCTCCCCCT (position 1662); GluR6 antisense, CACCAAATGCCTCCCACTATC (position 1921); GluR7 sense, TGGAACCCTACCGCTACTCG (position 713); GluR7 antisense, ACTCCACACCCCGACCTTCT (position 1115); KA-1 and KA-2 sense, TGGGCCTTCACCTTGATCATCA (position 1875 and 1872 for KA-1 and KA-2, respectively); KA-1 and KA-2 antisense, CTGTGGTCCTCCTCCTTGGG (position 2387 and 2384 for KA-1 and KA-2, respectively). To the 10 μl reverse transcription reaction (final deoxyribonucleotide concentrations, 50 μM each), 2.5 U of Taq polymerase (Stratagene) in the buffer supplied by the manufacturer were added to a final volume of 70 μl. Two drops of mineral oil were added, and after 3 min at 94°C, 30 μl containing the four primer pairs (10 pmol each) were included. Then, 20 cycles (94°C, 30 s; 50°C, 30 s; 72°C, 30 s) of PCR were performed followed by a final elongation period of 5 min at 72°C. The product of the first amplification was purified using GlassMAX spin cartridges (BRL), following the instructions of the manufacturer, and eluted in 45 μl. The purified first PCR (1 μl) product was used as a template in the second round of PCR amplification. In this second round, each subunit was individually amplified using its specific primer pairs and the same PCR program but with 35 cycles instead of 20. At the end of the second round, each cell resulted in four PCR products (subunit specific). Each amplification reaction product (7 μl) was run on a 2% agarose gel in parallel with ΦX174, HaeIII digested as molecular weight marker, and stained with ethidium bromide. The predicted sizes of the PCR-generated fragments were 208, 259, 421, and 512 bp for GluR5, GluR6, GluR7, KA-1, and KA-2 subunits, respectively.

Southern Blot Analysis

The GluR5, GluR6, GluR7, KA-1, and KA-2 PCR-generated fragments for all the cells were resolved independently on four different gels. Each gel was transferred onto Hybond N$^+$ (Amershan), and the Southern blots were hybridized with the following specific ^{32}P-labeled oligoprobes: GluR5 probe, GTGTCTTCTCCTTCCTCAACCCCCTATCTC (position 1667); GluR6 probe, GGCTTGCTTGGGTGTCAGTTGTGTGCTCT (position 1817); GluR7 probe, GCCCTGCTCTACCGATGCGGTCCACA (position 877); KA-1 and KA-2 probe, AAGCAGCCCAGCGTGTTTGTGAAGAGCACA (position 2088 and 2085 for KA-1 and KA-2 respectively; two mismatches with KA-2 subunit).

RNA Editing Analysis

To analyze the I/V and Y/C editing sites located in the first transmembrane domain of GluR6 subunit, 0.5 μl of the PCR-generated GluR6 fragment derived from each cell was dot blotted onto Hybond N$^+$ (Amersham) and hybridized with specific ^{32}P-labeled oligoprobes designed for the detection of the single nucleotide exchange to the edited and unedited versions of both sites. The following oligonucleotides were used. Site I/V: oligo V, AGCCAGCAGAACATACATCCA; oligo I, AGCCAGCAGAATATACATCCA. Site Y/C: oligo C, ACACCCAAGCAAGCCAGCAGA; oligo Y, ACACCCAAGTAAGCCAGCAGA. The temperatures of dissociation of the different oligonucleotides calculated using the Oligo program (Rychlik and Rhoads, 1989) were 64.8°C and 61.8°C for V and I oligonucleotides, respectively, and 71.9°C and 66.5°C for C and Y oligonucleotides, respectively. The hybridizations were performed overnight in 5× SSC, 1% SDS at 59.5°C and 64.5°C for the I/V and Y/C sites, respectively. The membranes were washed with 0.5× SSC, 0.1% SDS for 15 min at room temperature and exposed for autoradiography. The Q/R site located in the second transmembrane domain of the GluR6 subunit was analyzed by digesting the cDNA with the restriction enzyme AciI. We took advantage of the fact that the A to G change, operated by RNA editing, generates a restriction site for this endonuclease. Thus, the AciI restriction enzyme cuts the edited version (coding for R) and does not cut the unedited version (coding for Q). The digestion of the PCR-generated GluR6 fragment (259 bp) predicts two fragments of 62 and 197 bp. Both methods were tested by sequencing the GluR6 PCR-generated fragment before applying them to the cultured hippocampal neurons. For this purpose, the PCR-generated GluR6 fragment was subcloned using the TA cloning method (Invitrogen), following the instructions of the manufacturer. The colonies were transfered onto a filter for hybridization with the oligos I, V, C, and Y or cultured for plasmid purification and digestion with the AciI restriction enzyme. Then, seven clones were analyzed by DNA sequencing, confirming both the results obtained by hybridization and restriction enzyme digestion.

RNA Isolation and cDNA Preparation

Total RNA was prepared from fresh whole brain of 21-day-old rats by using the guanidinium thiocyanate single-step method (Chomczynski and Sacchi, 1987). The same procedure of reverse transcription and mPCR as described for single cells was applied to 1 ng total RNA.

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